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Comparison of Methods for Detection of Vaccinia Virus in Patient Specimens

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We analyzed a shell vial culture assay (SVA), real-time PCR, and a direct fluorescent antibody assay (DFA) for rapid detection of vaccinia virus from vaccination sites of Dryvax vaccine recipients. Of 47 samples assayed, 100% were positive by PCR, 89% were positive by SVA, and 40% were positive by DFA. DFA was limited by the need for adequate numbers of cells, with 32% of samples inadequate for interpretation. DFA performed better with specimens from patients who had not previously received the vaccine. PCR was positive for longer times postvaccination than was SVA. Infectious virus could be recovered after 45 min of acetone fixation of shell vial coverslips. Commercially available polyclonal antibodies cross-reacted with other orthopoxviruses and herpes simplex 1, but commercially available monoclonal antibodies were specific for vaccinia virus. In summary, PCR was the most sensitive test for detecting vaccinia virus in clinical specimens, while the DFA was the most rapid but the least sensitive test.

Vaccination with vaccinia virus poses some risk of complications that were well described for large surveys in the 1960s (13, 20). There are six well-recognized, serious complications of vaccinia inoculation: inadvertent inoculation (including ophthalmic vaccinia), generalized vaccinia, erythema multiforme, eczema vaccinatum, progressive vaccinia, and postvaccinia encephalitis. Complications due to vaccination with vaccinia virus must be differentiated from other illnesses presenting with a rash, including smallpox, varicella-zoster virus (VZV), herpes simplex virus (HSV), or enterovirus infection, allergic dermatitis, or drug rashes.

Most prior studies have used conventional cultures (3, 9, 17, 26) or PCR (3, 9, 10, 17) for detection of vaccinia virus in patient specimens. Some reports have described the use of direct fluorescent antibody methods for detection of vaccinia virus (9, 12, 16, 22) in a variety of specimens. Here we report the first use of a shell vial assay to detect vaccinia virus in specimens from vaccination sites and compare the shell vial assay with direct fluorescent antibody (DFA) testing and a real-time PCR assay. We find that the shell vial assay is usually positive within 18 to 24 h and is more sensitive than DFA.

MATERIALS AND METHODS

Patient specimens. Specimens were obtained from the inoculation sites of 13 individuals who had voluntarily agreed to be vaccinated with the Dryvax smallpox vaccine through a vaccination program offered to the employees of the National Institutes of Health. Specimen collection was part of a protocol approved by the Institutional Review Board of the Warren G. Magnuson Clinical Center. For seven individuals this was a revaccination, and for six this was their primary vaccination with Dryvax. Specimens for shell vial culture and PCR were obtained on swabs, placed in Bartels ViraTrans transport medium (Trinity Biotech, Wicklow, Ireland), and transported to the laboratory on ice. For PCR, a 200-μl aliquot of the transport medium was placed into a microcentrifuge tube containing 0.9 ml lysis buffer, which is included in the NucliSens isolation kit (bioMerieux, Inc., Durham, NC), and was stored at −70°C until DNA was extracted. Specimens for DFA from the inoculation site were obtained using a tongue depressor and placed on a microscope slide. Vaccination sites were covered with an occlusive dressing, and specimens from the vaccination sites were collected when this dressing needed to be changed due to accumulation of fluid or pus at the vaccine site or because the dressing had become loose. The decision to change the dressing was made by a clinician.

Antibodies. Fluorescein isothiocyanate (FITC)-conjugated rabbit antibodies to vaccinia virus were purchased from Biodesign International (Saco, ME), Fitzgerald Industries International, Inc. (Concord, MA), and ViroStat (Portland, ME) and were diluted in 0.005% Evans blue–phosphate-buffered saline (PBS). The specification sheets for the antibodies indicated that they do not cross-react with parainfluenza (1–3), respiratory syncytial virus, adenovirus, influenza A or B, or HSV type 1 (HSV-1). We also evaluated two unconjugated mouse monoclonal antibodies to vaccinia virus (Biodesign International) used in conjunction with FITC anti-mouse antibodies (Trinity Biotech plc, Wicklow, Ireland).

Cell lines. HeLa 229, MRC-5, A549, Mink Lung, Hep2, Vero, RhMK, and CHO cell monolayers on coverslips in shell vials were purchased from Diagnostic Hybrids, Inc., Athens, OH. MRC-5 cells were also purchased in T150 flasks (Diagnostic Hybrids). B78H1 mouse melanoma cells (a gift from Nigel Frasier, University of Pennsylvania) were removed from T80 flasks by the use of trypsin, suspended in growth medium, and used to seed shell vials containing circular coverslips.

Cell culture medium. Shell vial maintenance medium consisted of RPMI 1640 (Sigma, St. Louis, MO) or Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 1% fetal bovine serum (Biowhittaker, Walkersville, MD) and a penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) mix (Biowhitaker). Growth medium contained 6% fetal bovine serum.

Vaccinia virus. The Wyeth strain of vaccinia virus was kindly provided by Bernard Moss of the National Institute for Allergy and Infectious Diseases, Bethesda, MD.

Additional viruses. Inactivated camelpox, monkeypox, and cowpox viruses were supplied fixed on glass microscope slides by Inger Damen at the Centers for Disease Control. These prepared slides had been gamma irradiated with 4.4 rads (equivalent to one kill cycle). To obtain virus for PCR, virus-infected and control cells were removed from the Centers for Disease Control-prepared slides. A 50-μl aliquot of sterile PBS was placed onto the individual slide spot, and a wooden applicator stick was used to scrape the cells from the slide and suspend them in PBS. The aliquot was transferred to a microcentrifuge tube that con-

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tained 0.9 ml of lysis buffer (bioMerieux, Inc.). Patient isolates of HSV-1, HSV-2, and VZV cultivated in shell vials containing MRC-5 cells were used to test the antibodies for cross-reactivity.

Shell vial assay. The shell vial assay was performed using the same procedure used in the clinical virology laboratory in the National Institutes of Health Warren G. Magnuson Clinical Center for respiratory viruses, cytomegalovirus, HSV, and VZV (4). Briefly, culture medium was removed from duplicate shell vials and 200 μl of specimen was placed over the monolayer of cells. Virus titer endpoints were determined by limiting dilution using 1:10 serial dilutions in cell culture medium. Inoculated shell vials were centrifuged at 3,500 × *g* for 15 min at 25°C in a Centra-8R refrigerated centrifuge (International Equipment Co., Needham Heights, MA). Following centrifugation, 0.5 ml of cell culture medium was added to each shell vial and inoculated shell vials were incubated at 35°C for 18 to 24 or 48 h. The monolayers on the shell vial coverslips were fixed in 1 ml of acetone for 10 min, washed with PBS, and stained with FITC-conjugated polyclonal antibodies in Evans blue. Coverslips were mounted on glass slides in Bartels buffered glycerol mounting medium (Trinity Biotech) and examined for fluorescent foci with a Zeiss fluorescence microscope. RhMK cells that had been inoculated with 10-fold dilutions of Wyeth vaccinia strain stock and incubated for 24 h were used to determine the effect of acetone fixation on vaccinia virus infectivity. One pair of vials for each dilution (10^{-2} to 10^{-7}) was fixed and stained as described above. A third vial for each dilution was fixed using 5.5 ml (shell vial filled to the top) acetone for 10 min at room temperature, the monolayers were washed once with PBS, 0.5 ml feeding medium was added to each vial, and the cell monolayers were scraped off using a plastic pipette. The suspensions of scraped cells were subjected to sonication, two fresh RhMK shell vials for each dilution were inoculated with 200 μl of sonicate, and the remainder of the shell vial assay was performed as described above. This experiment was repeated with infected RhMK monolayers exposed to acetone for 15, 30, 45, and 60 min at room temperature.

Plaque assay for vaccinia virus. The sensitivity of the shell vial assay using HeLa 229 cells was evaluated by comparing the same 1:10 serial dilution series inoculated in a plaque assay. HeLa 229 cells were plated in six-well dishes in DMEM with 10% fetal bovine serum and penicillin and streptomycin. The following day, the medium was removed and serial dilutions of vaccinia virus were added to the wells in duplicate in a total volume of 1 ml. After incubation for 2 h at 37°C, the wells were overlaid with 3 ml of DMEM with 2.5% fetal bovine serum and penicillin and streptomycin. After 2 days the medium was removed and plaque assay dye (0.1% crystal violet in formaldehyde and methanol) was added to the wells. The dye was removed after 1 h, plaques were counted, and the titer was determined. Large plaques, not satellite plaques, were counted.

Host cell disruption methods. Methods of host cell disruption as described by Blattner et al. (1) were used to determine the specimen processing method that would provide the highest yield of vaccinia virus in the shell vial assay. Infected HeLa 229 cells were harvested using glass beads and vortex mixing as described above without disruption. The pelleted cells were resuspended in 4 ml of Bartels ViraTrans medium (Trinity Biotech) and then distributed into four 1-ml aliquots to be subjected to four different cell disruption methods: vortex mixing for 1 min with three glass beads, three freeze-thaw cycles using dry ice, sonication for 1 min using a Heat Systems cup horn sonicator (Farmingdale, NY), and homogenization in Genetron for 2 min using a bead beater (Biospec Products, Bartlesville, OK). Shell vials containing monolayers of RhMK cells were used to determine extinction dilution endpoints.

Direct fluorescent antibody assay. Smears were fixed in acetone for 10 min, air dried, and stained for 30 min at 35°C with 100 μl of FITC-conjugated polyclonal antibodies in Evans blue. After washing in PBS, coverslips were mounted on glass microscope slides in Bartels buffered glycerol mounting medium (Trinity Biotech) and examined for fluorescent foci with a Zeiss fluorescence microscope. Specimens were considered to be of a quantity not sufficient (QNS) when less than 1 cell per 40× field was present and no vaccinia virus-infected cells were noted.

PCR. DNA was prepared from cell lysates by use of a NucliSens isolation kit. To verify that PCR inhibitors were removed during DNA extraction, an internal control amplifiable by *pan*-orthopox primers was constructed using previously described procedures (6). A plasmid that contained the amplification site was generated and diluted to 5 to 5,000 copies/reaction. Real-time PCR was performed on a LightCycler instrument (Roche Molecular Biochemicals, Indianapolis, IN). *Pan*-orthopox virus primers OPE9L-F1880 and OPE9L-R2057 (11) were used to amplify a 177-bp region of the DNA polymerase-E9L genes. Each reaction tube contained 10 μl of master mix, a 10-μl aliquot of the extracted DNA, and probe set (labeled with Red 640) E9L.FRET.up (5′-CGTATA TTG CAT GGA ATC ATA GAT GGC CTT T-fluorescein-3′) and E9L.FRET.dn (5′-Red 640-AGT TGA ACT GGT AGC CTG TTT TAA CAT CTT T-3′). For internal control detection, a probe set labeled with Red 705 as the reporter was used: Mimic-FRET.1 (5′-GAT ATC GTC CAT TCC GAC AGC ATC-fluorescein-3′) and Mimic-FRET.2 (5′-Red 705-CCA GTC ACT ATG GCG TGC TGC TAG-3′). The PCR protocol consisted of a 10-min incubation at 30°C and a 10-min incubation at 95°C, followed by a touch-down procedure that consisted of 5 s at 95°C, annealing for 10 s at temperatures decreasing from 66°C to 54°C during the first 12 cycles (with 1°C decreasing steps for each cycle), and an extension step at 72°C for 20 s (14). The annealing temperature for the remaining 34 cycles was 54°C for 10 s. Under these conditions the PCR detected 25 copies per ml of vaccinia virus.

RESULTS

Cross-reactivity of vaccinia virus antibodies. FITC-conjugated polyclonal antibodies from all three vendors had identical performance characteristics in our laboratory. The antibodies had an optimal working dilution of 1:50 for both shell vial cultures and DFA. As expected, the vaccinia virus antibodies cross-reacted with monkeypox, camelpox, and cowpox in DFA performed on fixed smears. Surprisingly, these antibodies cross-reacted with HSV-1 (but not HSV-2 or VZV). In contrast, the anti-vaccinia monoclonal antibodies did not cross-react with other poxviruses, HSV-1, HSV-2, or VZV and therefore were more specific for detection of vaccinia.

Vaccinia viruses grew similarly at 35°C for 24 and 48 h in HeLa 229, MRC-5, A549, Mink Lung, HEp2, Vero, RhMK, and B78H1 cells but grew very poorly in CHO cells. B78H1 cells were evaluated because they are nonpermissive for HSV infection (18). Thus, B78H1 cells could be used for detection of vaccinia virus with the FITC-conjugated polyclonal anti-vaccinia antibodies in clinical specimens, since vaccinia virus, but not HSV, would grow in the cells and therefore cross-reactivity with HSV should not be a problem.

Methods to enhance virus recovery. Centrifugation of the inoculum onto shell vial HeLa 229 cell monolayers increased the sensitivity of the assay by 100-fold compared to uncentrifuged vial results. The uncentrifuged viral limiting dilution endpoint was 10^{-4}, and the centrifuged endpoint was 10^{-6}. Homogenization of virus-infected cells in Genetron, or sonication of the cells, also improved virus recovery. The limiting dilution endpoints of virus, as measured by shell vial assay after 24 h of incubation, were 10^{-5} after freezing-thawing or vortexing infected cells versus 10^{-7} after sonicating the cells or homogenizing the cells in Genetron. After 48 h the limiting dilution endpoints were 10^{-5} after freezing-thawing infected cells, 10^{-6} after vortexing cells, and 10^{-7} after sonication or homogenization in Genetron. The shell vial assay and the conventional plaque assay for vaccinia virus both detected ≥4 PFU of virus.

Vaccinia virus is infectious after acetone fixation. The limiting dilution endpoint for the primary inoculum that was fixed and stained on shell vial coverslips after 24 h was 10^{-7}. Infectious virus was detected after exposure to acetone for 10 min in all dilutions up to 10^{-6}. Fixation for 10 min in acetone is the standard fixation step for all shell vial assays performed in our laboratory. Infected shell vials were exposed to acetone for 15, 30, 45, and 60 min to determine how long shell vial monolayers infected with vaccinia virus must be exposed to acetone to completely inactivate all infectious virions. In the control shell vials the entire monolayer was infected at the 10^{-2} dilution, and a mean of 17 fluorescent foci was observed at the 10^{-6}

TABLE 1. Results for shell vial assay (SVA), DFA, and PCR performed on specimens obtained from vaccination sites of volunteer vaccinees who had received the vaccine sometime previously

Patient	Assay	Result at indicated day(s) postvaccination[a]						
		4	6–7	10–11	14–17	18–19	27–28	29–34
1	DFA	QNS	−	−				
	SVA	+	+	−				
	PCR	+	+	+				
2	DFA	ND	+	QNS	QNS			
	SVA	ND	+	+	+			
	PCR	ND	+	+	+			
3	DFA	ND	QNS	−				
	SVA	ND	+	+				
	PCR	ND	+	+				
4	DFA	ND	−	−				
	SVA	ND	+	+				
	PCR	ND	+	+				
6	DFA	ND	+	QNS	+	QNS	QNS	−
	SVA	ND	+	+	+	+	+	−
	PCR	ND	+	+	+	+	+	+
8	DFA	ND	+	QNS	−	QNS	ND	−
	SVA	ND	+	+	+	+	ND	−
	PCR	ND	+	+	+	+	ND	+
11	DFA	ND	ND	ND	ND	+	QNS	ND
	SVA	ND	ND	ND	ND	+	+	−
	PCR	ND	ND	ND	ND	+	+	−

[a] QNS, quantity not sufficient; ND, not done; +, positive; −, negative.

dilution. Shell vials inoculated with infected cells that had been exposed to acetone for 15 to 60 min had fluorescent foci observed in the following dilutions: 10^{-5} for 15 min of exposure, 10^{-4} dilution for 30 min of exposure, and 10^{-3} for 45 min of exposure. No fluorescent foci were observed in any dilution for 60 min of exposure.

Comparison of detection methods for vaccinia virus in patient specimens. Vaccinia virus was detected by shell vial assay, DFA, and PCR from the vaccination sites of 13 volunteer vaccinees (Tables 1 and 2). Both the shell vial assay and PCR were more sensitive than DFA ($P < 0.0001$ [t test for correlated samples]). Of the 47 samples tested both by shell vial assay and DFA, 89% (42/47) were positive by shell vial, while 40% (19/47) were positive by DFA and 32% (15/47) had inadequate numbers of cells for the DFA to be interpreted. Of the 42 samples that were positive by shell vial, 38 (90%) were positive at 18 to 24 h, and 4 (10%) required 48 h to become positive. PCR was more sensitive than DFA ($P < 0.0001$ [t test for correlated samples]), with 47 (100%) of 47 specimens positive by PCR, but the corresponding DFA specimens were positive in only 19 (40%) of the 47 specimens.

PCR was more sensitive than shell vial culture ($P = 0.0009$ [t test for correlated samples]). Both PCR and shell vial assays were performed on 53 specimens; PCR was positive for 51 (96%) specimens, whereas the shell vial assay was positive in 42 (79%) specimens. The two PCR-negative specimens were from inoculation sites 33 and 34 days postvaccination. In specimens from eight patients the PCR was positive for a longer time postvaccination than was culture.

DFA performed on specimens from primary vaccinees was more likely to be positive than DFA performed on specimens from individuals who had been vaccinated sometime in the past. For primary vaccinees the DFA was positive for 58% (14/24) of the samples, with 21% (5/24) with too few cells (Table 2), and for specimens from previously vaccinated volunteers the DFA was positive for only 22% (5/23), with 43% (10/23) specimens with too few cells (Table 1).

DISCUSSION

This is the first description of the application of the shell vial assay for detection of vaccinia virus in patient specimens. We found that vaccinia virus can be detected in most clinical specimens at 18 to 24 h by use of the shell vial assay and that a wide variety of commonly used cell lines are equally effective for isolation of vaccinia virus. The shell vial assay was more sensitive than DFA but less sensitive than PCR. Three FITC-conjugated polyclonal antibodies from different vendors were found to cross-react with HSV-1 but not with HSV-2 or varicella-zoster virus in the shell vial assay. These antibodies also detected other orthopoxviruses (monkeypox, camelpox, cowpox) on fixed microscope slides. The two monoclonal antibodies we tested were specific for vaccinia virus. Technical data from Biodesign International indicate that the monoclonal antibody reacts with a 27-kDa protein in the Western blot assay.

We performed DFA for vaccinia on cell smears from inoculation sites of vaccinees. While DFA results were available in a few hours, for one-third of specimens there were inadequate numbers of cells available for performing DFA. In 1976 Maltseva and Nishanov described the use of polyclonal antibodies in a DFA for rapid diagnosis of diseases caused by poxviruses (16). They reported that the results of examination of smears from pustular fluid and scabs were not reliable in 44% of cases due to the presence of nonspecific fluorescence of tissue fragments and leukocytes. Only 34% (12/35) of smears from late postvaccination stages were positive by DFA, whereas 100% (22/22) of smears from early stages (maculopapular and vesicular) were positive. We did not observe nonspecific fluorescence of tissue fragments and leukocytes in our study and had

TABLE 2. Results for shell vial assay (SVA), DFA, and PCR performed on specimens obtained from vaccination sites of volunteer vaccinees for whom this was their primary vaccination

Patient	Assay	Result at indicated day(s) postvaccination[a]									
		6–7	10–11	11–16	17–20	23–24	26–28	29–31	33–39	43	49
5	DFA	−	+	+	ND	ND	ND				
	SVA	+	+	+	ND	ND	−				
	PCR	+	+	+	ND	ND	+				
7	DFA	−	ND	+	ND	ND	+	ND	ND		
	SVA	+	ND	+	ND	ND	+	−	−		
	PCR	+	ND	+	ND	ND	+	+	−		
9	DFA	+	−	+	ND	+	ND	ND			
	SVA	+	+	+	ND	+	ND	−			
	PCR	+	+	+	ND	+	ND	+			
10	DFA	+	+	+	QNS	+	ND	QNS			
	SVA	+	+	+	+	+	ND	−			
	PCR	+	+	+	+	+	ND	+			
12	DFA	ND	ND	ND	QNS	+	+	ND	ND	+	ND
	SVA	ND	ND	ND	+	+	+	ND	ND	−	−
	PCR	ND	ND	ND	+	+	+	ND	ND	+	+
13	DFA	QNS	−	QNS	ND	ND	ND	−			
	SVA	+	+	+	ND	ND	ND	+			
	PCR	+	+	+	ND	ND	ND	+			

[a] QNS, quantity not sufficient; ND, not done; +, positive; −, negative.

no false-positive DFA results. More recent studies have reported the use of DFA for detection of vaccinia in brain biopsies and in conjunctival scrapings (49, 12). We found DFA to be more frequently positive with specimens from individuals who had received the Dryvax vaccine for the first time. For these individuals, DFA was positive in 58% of specimens (Table 2) versus a positive rate of only 22% for previously vaccinated individuals. Primary vaccination is usually associated with the development of a vesicle followed by a pustule at the vaccine site, while revaccination is often associated with induration rather than a pustule (8). Thus, it is more difficult to obtain adequate numbers of cells to perform a DFA in lesions from vaccinated persons.

Our real-time PCR detected vaccinia virus for a longer duration after vaccination of eight patients than did shell vial culture. However, it is not certain that viral DNA detected at these times reflects the presence of infectious virus. The primer pair used in our real-time PCR using fluorescence resonance energy transfer probes is very sensitive and is specific for the DNA polymerase gene of all orthopoxviruses as described by Kulesh et al. (11). Real-time PCR assays using fluorescence resonance energy transfer probes have recently been described by Espy et al. (5) and Panning et al. (24) by use of primers for the orthopox virus hemagglutinin gene and by Nitsche et al. (21) by use of primers for the VTEF gene. These targets are found in all orthopox viruses and have enough sequence variability that melting analysis following PCR enabled the identification of different orthopox viruses by the characteristic melting temperatures of the PCR products. The region of the DNA polymerase-E9L genes amplified in our real-time PCR is conserved among the different orthopoxviruses, and therefore we cannot distinguish between different orthopoxviruses with our PCR. We are developing different real-time PCR assays specific for different orthopoxviruses to be used for virus identification.

We detected infectious vaccinia virus after 45 (but not 60) min of acetone fixation of our shell vial monolayers. Potajallo et al. reported in 1968 that both the WR strain of vaccinia virus and the Harvey strain of variola virus could be inactivated after 45 min of acetone treatment (25). This finding emphasizes the need for laboratories that perform shell vial assays, other culture assays, or DFA for vaccinia virus to follow the most current recommendations from the Centers for Disease Control (2). This is underscored by the numerous reports of laboratory-acquired infections with vaccinia virus (7, 15, 17, 19, 23, 27). Vaccination with vaccinia virus is recommended for laboratory workers who directly handle (i) cultures or (ii) animals contaminated or infected with non-highly-attenuated vaccinia virus, recombinant vaccinia viruses derived from non-highly-attenuated vaccinia strains, or other orthopoxviruses that infect humans. Vaccination is not recommended for persons who do not directly handle non-highly-attenuated vaccinia virus cultures or materials. Vaccinia virus is currently not a select agent, and diagnostic laboratory procedures involving vaccinia virus can be performed in a biosafety level 2 laboratory with a class II biological safety cabinet.

We conclude that the shell vial assay for culture of vaccinia is useful for isolation of vaccinia virus from patients with complications due to smallpox vaccination. Culture may be useful to determine whether a lesion or dressings are infectious or to obtain an isolate for antiviral susceptibility testing or for further characterization. The use of DFA can provide a result in a few hours when the specimen is properly collected and is more likely to be positive in primary vaccinees. Indirect immunofluorescence with vaccinia virus monoclonal antibodies (which do not cross-react with HSV-1 or other poxviruses) could also be used in the shell vial assay or directly on smears from lesions to increase specificity. Real-time PCR provides an alternative assay for the presence of orthopoxviruses in patient specimens, because it is rapid, specific, and more sensitive than shell vial culture and DFA.

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